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Effects of Trypsin, Protease Inhibitors and Ethanol on Corpus Luteum Adenylyl Cyclase[1]

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ABSTRACT

The effects of trypsin, 2 protease inhibitors (N-alpha-p-tosyl-L-lysine chloromethyl ketone HCl [TLCK] and L-1-tosylamide-2-phenylethyl chloromethyl ketone [TPCK]) and ethanol on rabbit and rat corpus luteum adenylyl cyclase were studied. Trypsin stimulated adenylyl cyclase activity in rabbit corpus luteum membranes. Maximal stimulation was observed at 2.5 μg/ml trypsin. Higher trypsin concentrations inhibited rabbit corpus luteum adenylyl cyclase activity. Addition of 10 μM GTP did not alter the activation of adenylyl cyclase by trypsin. In the presence of ethanol, TLCK inhibited hCG and LH stimulation of rabbit and rat corpus luteum adenylyl cyclase. Ethanol, alone, enhanced hCG and LH stimulation of adenylyl cyclase up to a concentration of 5% ethanol, while higher concentrations of ethanol inhibited hCG and LH stimulation of ovarian adenylyl cyclase. These responses in the rat ovarian adenylyl cyclase were not altered by GTP; in the rabbit corpus luteum adenylyl cyclase, TLCK in the presence of ethanol and GTP actually enhanced hCG and LH stimulation of adenylyl cyclase up to a concentration of 0.625 mM TLCK. Higher concentration of TLCK in the presence of ethanol and GTP inhibited hCG and LH stimulation of the rabbit enzyme. The combined effects of ethanol and TLCK on ovarian adenylyl cyclase do not appear to involve alteration of the apparent affinity of hCG for its receptor and are due primarily to a reduction in the maximal velocity of the reaction. It appears that TLCK may be enhancing the inhibitory action of high concentrations of ethanol on hCG and LH stimulation of ovarian adenylyl cyclase. Based on the findings of the present study, it would appear to be premature to propose a central role for proteases in hCG and LH stimulation of ovarian adenylyl cyclase.

INTRODUCTION

Recent studies have suggested an integral role for proteases in the activation of adenylyl cyclase by hCG in the rat ovary (Richert and Ryan, 1977a,b). The proposed involvement of proteases in hCG stimulation of rat ovarian adenylyl cyclase is based on the findings that serine proteases stimulated rat ovarian adenylyl cyclase and that hCG stimulation of ovarian adenylyl cyclase could be blocked by synthetic protease inhibitors. Due to the importance of the adenylyl cyclase system in the regulation of polypeptide hormone action and the implication of proteases in regulating this enzyme system, we reexamined the action of trypsin and 2 protease inhibitors on the stimulation of ovarian adenylyl cyclase in the rabbit and rat. Based on the findings of the present study, we feel it is premature to suggest an integral role for proteases in hormonal stimulation of adenylyl cyclase.

MATERIALS AND METHODS

Animals

New Zealand White rabbits (3.5–4.5 kg BW) were used. Pregnancy was induced by mating female rabbits with experienced bucks. The rabbits were sacrificed by nembutal injection on Day 5 of pregnancy. The ovaries were removed and placed in ice cold Krebs Ringer bicarbonate (KRB), pH 7.4, until dissection of the corpora lutea.

Prepubertal Sprague-Dawley-derived female rats were received at 23 days of age and "superovulated" by injection at 25 days of age with PMSG (50 IU, s.c., 0900 h) and 56 h later with hCG (50 IU, s.c.). Superovulated rats were sacrificed by decapitation 7 days after hCG injection. The ovaries were removed, trimmed and kept in ice cold KRB until homogenization. The dissected rabbit corpora lutea and rat ovaries were homogenized and membrane particles prepared as described previously (Birnbaumer et al., 1976).

Adenylyl Cyclase Assay

Adenylyl cyclase activity was determined in a final volume of 50 μl containing 3.0 mM ATP (with 7–10

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$\times 10^6$ CPM of [α-^{32}P]-ATP), 5.0 mM $MgCl_2$, 1.0 mM EDTA, 1.0 mM cAMP (with ~10,000 CPM [^{3}H]-cAMP), 20 mM creatine phosphate, 0.2 mg/ml creatine kinase, 0.02 mg/ml myokinase and 25 mM Tris HCl buffer, pH 7.5. Incubations were carried out at 32.5°C for 10 min. The reaction was stopped with 0.1 ml of solution containing 40 mM ATP, 10 mM cAMP and 1% sodium dodecylsulfate, followed by immediate boiling for 3.5 min. The [^{32}P]-cAMP formed and [^{3}H]-cAMP added to monitor recovery were isolated according to the method of Salomon et al. (1974) using Dowex and alumina chromatography with minor modifications described elsewhere (Bockaert et al., 1976).

Protein was determined by the method of Lowry et al. (1951), using crystalline bovine serum albumin (fraction V) as standard.

Materials

The LH (NIH-LH-S18) and highly purified hCG (hCG-CR 119) were gifts from the Endocrine Study Section, NIH. The hCG used to induce superovulation was a gift from Ayerst. The PMSG, L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK) and N-alpha-p-tosyl-L-lysine chloromethyl ketone HCl (TLCK) were purchased from Sigma Chemical Co. Trypsin was purchased from Worthington. The sources of the materials used in the adenylyl cyclase assay were as described previously (Birnbaumer et al., 1976).

Fresh inhibitor solutions were prepared just prior to use. Both TPCK and TLCK were dissolved in absolute ethanol at a concentration of 50 mM. The protease inhibitors and ethanol controls were diluted in 0.1% bovine serum albumin prior to addition to the assay.

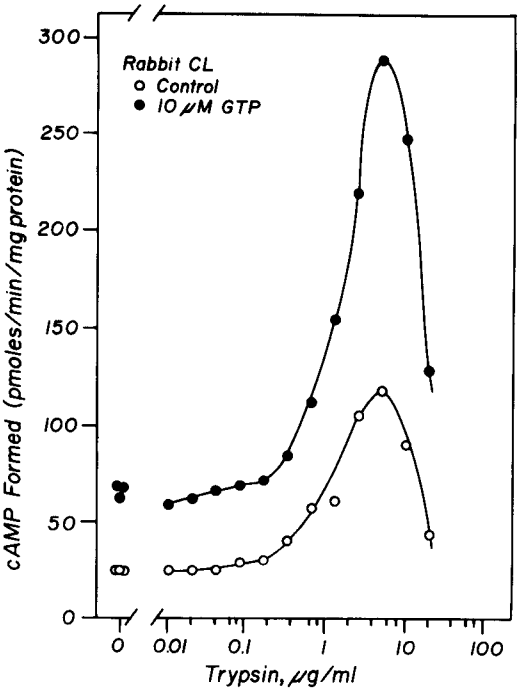

FIG. 1. Effect of trypsin on adenylyl cyclase activity in membranes from rabbit corpora lutea. Membrane particles from rabbit corpora lutea were incubated as indicated under Materials and Methods in the presence of the indicated concentrations of trypsin. The trypsin concentration was vaired from 0.01 to 20 μg/ml at 2-fold intervals for a total of 12 concentrations. When present, GTP was 10 μM. Membrane protein was 15.0 μg/assay.

RESULTS

Proteolytic Activation of Rabbit Corpus Luteum Adenylyl Cyclase

Basal and GTP stimulated rabbit corpus luteum adenylyl cyclase activity was enhanced in the presence of trypsin (Fig. 1). Maximal stimulation of adenylyl cyclase activity was observed at 2.5 μg/ml trypsin, while higher concentrations depressed enzymatic activity. Activation of adenylyl cyclase by trypsin was not altered by GTP as 2.5 μg/ml trypsin caused a 4.8 and 4.4-fold stimulation of adenylyl cyclase activity in the absence and presence of 10 μM GTP, respectively.

Effect of Ethanol and Protease Inhibitors on LH- and hCG- induced Stimulation of Rabbit and Rat Corpora Luteal Adenylyl Cyclase

Due to the insolubility of both TPCK and TLCK in aqueous solution, it was necessary to dissolve these protease inhibitors in absolute ethanol. Therefore, appropriate ethanol controls were performed throughout. Ethanol alone stimulated adenylyl cyclase activity in the presence and absence of 10 μM GTP in both the rabbit and rat enzyme preparations (Figs. 2, 3; upper panels). Maximal stimulation with ethanol was observed at the highest concentration utilized (20%). In the rabbit corpus luteum membranes, GTP reduced the degree of maximal stimulation by ethanol from 7-fold in the absence of GTP to 3-fold in the presence of GTP (Fig. 2; upper panel). In the rat ovarian membranes, on the other hand, GTP did not alter the degree of maximal adenylyl cyclase stimulation by ethanol which was 2-fold (Fig. 3; upper panel). In the presence and absence of GTP, ethanol produced a biphasic alteration in the adenylyl cyclase response to LH and hCG (Figs. 2, 3). Up to a concentration of 5%,

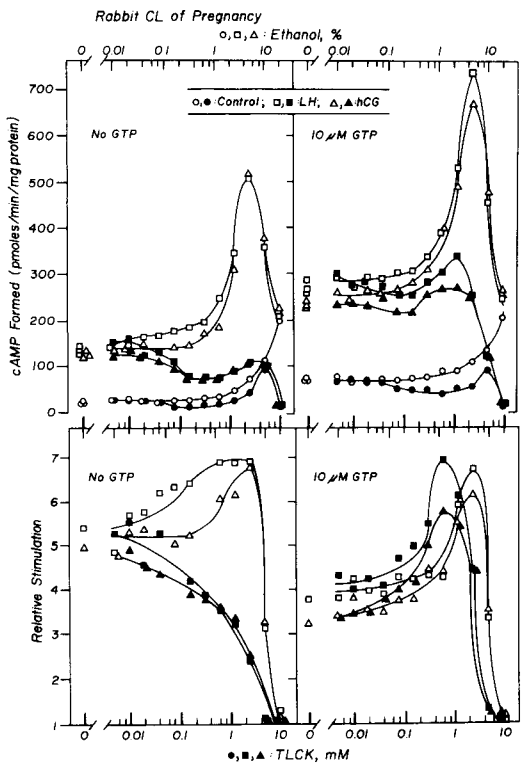

FIG. 2. Effect of ethanol and N-alpha-p-tosyl-L-lysine chloromethyl ketone HCl (TLCK) on the ability of LH and hCG to stimulate adenylyl cyclase in membranes from rabbit corpora lutea of pregnancy. Membrane particles from rabbit corpora lutea were incubated as indicated under Materials and Methods in the presence of the indicated concentrations of ethanol (open symbols), TLCK in ethanol (closed symbols), in the absence (○,●) or presence of LH (□,■; 1 μg/ml) or hCG (△,▲; 1 μg/ml). The ethanol and TLCK in ethanol concentrations were varied at 2-fold intervals for a total of 12 concentrations from 0.01–20% ethanol and from 5 μM–10 mM TLCK in 0.01–20% ethanol. When present, GTP was 10 μM. Membrane protein was 20.5 μg/assay. Relative stimulation was determined by dividing each experimental value by its appropriate control value.

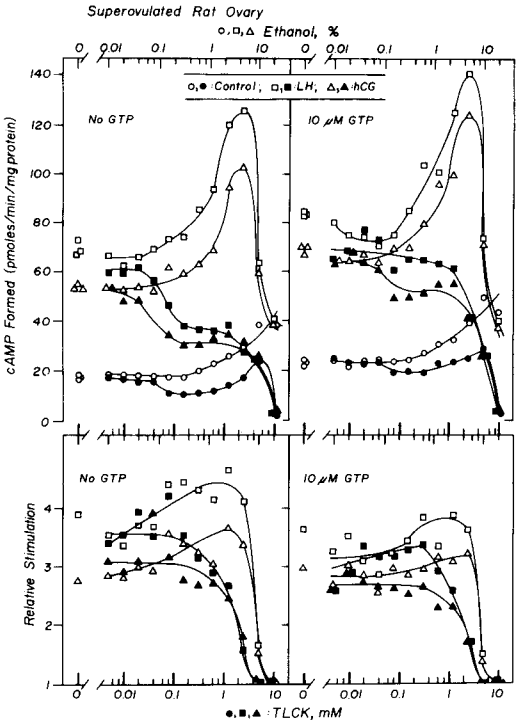

FIG. 3. Effect of ethanol and N-alpha-p-tosyl-L-lysine chloromethyl ketone HCl (TLCK) on the ability of LH and hCG to stimulate adenylyl cyclase in membranes from superovulated rat ovaries. Characterization conditions and symbols are the same as Fig. 2 except that membrane particles from superovulated rat ovaries were used and 48.5 μg of membrane protein was used/assay.

ethanol enhanced the adenylyl cyclase response to both gonadotropins. Above this concentration ethanol depressed the adenylyl cyclase response to the gonadotropins. Half-maximal inhibition of hCG and LH stimulation of ovarian adenylyl cyclase occurred at ∿10% ethanol in both enzyme preparations.

The TLCK, dissolved in ethanol, altered adenylyl cyclase activity in a manner similar to that of ethanol over the concentration range of 5–40 μM TLCK in both enzyme preparations (Figs. 2, 3). Above 40 μM, TLCK depressed adenylyl cyclase activity below its corresponding ethanol control. Maximal depression of adenylyl cyclase activity by TLCK occurred at the highest concentration of TLCK used (10 mM). The response to TLCK was not altered by GTP. This inhibited activity ranged between 7–15% of the control value. In the presence of LH or hCG, TLCK produced a complex response pattern (Figs. 2, 3). A similar pattern was observed with TPCK; however, upon addition to the assay, the TPCK came out of solution. Therefore, the data are not shown. In the absence of GTP, TLCK depressed LH and hCG stimulable adenylyl cyclase similarly in both the rabbit and rat enzyme preparations. Addition of 10 μM GTP did not alter this response in the rat ovarian adenylyl cyclase. In the rabbit enzyme, TLCK in the presence of GTP produced a biphasic response, enhancing LH and hCG stimulation up to a concentration of 0.625 mM TLCK and inhibiting hormonal stimulation

at higher TLCK concentrations. Half-maximal inhibition of LH and hCG stimulation of the rabbit and rat enzymes in the presence and absence of GTP was ∿2 mM TLCK which corresponds to 4% ethanol.

Because several serine proteases and the beta chain of LH and hCG contain an area of homology in their amino acid sequence (Kurosky et al., 1977), we examined the possibility that TLCK interacted with the hormone directly to inhibit LH and hCG stimulation of ovarian adenylyl cyclase. We determined whether the apparent affinity of hCG for its receptor was altered by TLCK and ethanol. In the presence of 10 μM GTP, hCG half-maximally stimulates rabbit corpus luteum adenylyl cyclase at a concentration of 60 ng/ml hCG. Neither ethanol alone nor ethanol plus TLCK significantly altered the concentration of hCG required to half-maximally stimulate the rabbit corpus luteum adenylyl cyclase (Fig. 4).

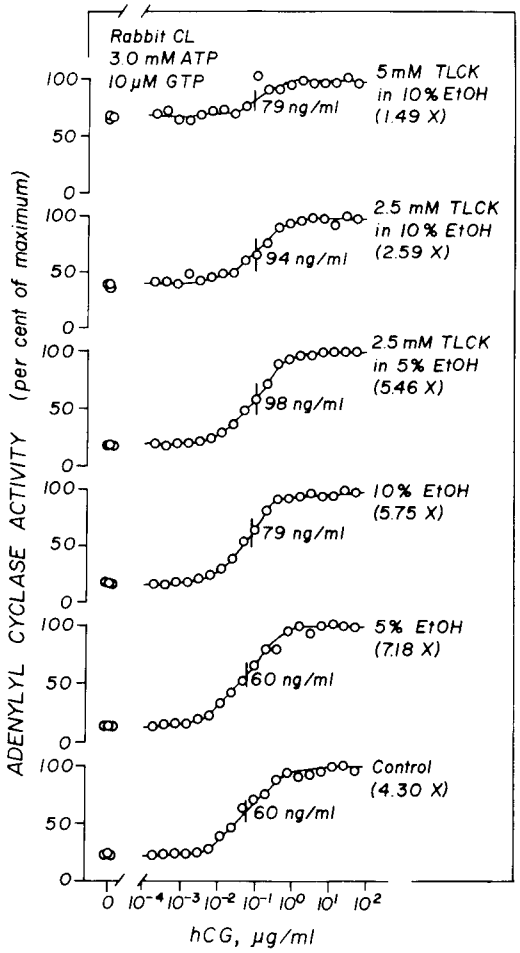

Fig. 4. Effect of ethanol and N-alpha-p-tosyl-L-lysine chloromethyl ketone HCl (TLCK) on concentration-effect curves for hCG on rabbit corpus luteum adenylyl cyclase. Membrane particles from rabbit corpora lutea were incubated as indicated under Materials and Methods in the presence of the indicated concentrations of hCG in the absence (control) or presence of the indicated concentration of ethanol or TLCK plus ethanol. The hCG concentration was varied from 0.2 μg/ml to 50 μg/ml at 2-fold intervals for a total of 19 concentrations. Basal activity in the control, 5% ethanol, 10% ethanol, 2.5 mM TLCK in 5% ethanol, 2.5 mM TLCK in 10% ethanol and 5 mM TLCK in 10% ethanol groups are 57, 74, 90, 44, 70 and 67 pmoles cAMP formed/min/mg protein, respectively. Membrane protein was 9.8 μg/assay. The numbers in parentheses represent the -fold stimulation by saturating concentrations of hCG.

DISCUSSION

In the present study, trypsin activates rabbit corpus luteum adenylyl cyclase. Similar findings in rat liver (Hanoune et al., 1977), luteinized rat ovary (Richert and Ryan, 1977a) and fibroblast (Anderson et al., 1978) adenylyl cyclase have led to the suggestion that proteolytic activation may be a means of regulating this enzyme system. Support for a possible central role of proteases in the activation of adenylyl cyclase came from studies with synthetic protease inhibitors (Richert and Ryan, 1977b). These protease inhibitors were reported to block hCG activation of ovarian adenylyl cyclase and it was suggested that the inhibitors did not interact with the hormone directly but blocked hCG stimulation of adenylyl cyclase after hCG had been bound to its receptor. The findings of the present study in which TLCK and ethanol did not alter the apparent affinity of hCG for its receptor would appear to support these conclusions of Richert and Ryan (1977b).

Although in both the present study and in the report by Richert and Ryan (1977b) synthetic protease inhibitors inhibited hCG stimulation of adenylyl cyclase, an alternate explanation for these results is possible. In the present study, high concentrations of ethanol inhibited LH and hCG stimulation of ovarian adenylyl cyclase with half-maximal inhibition occurring at ∿10% ethanol. Half-maximal inhibition of LH and hCG stimulation of adenylyl cyclase by TLCK occurs at ∿2 mM TLCK which corresponds to an ethanol concentration of 4%. Thus, it appears that TLCK is enhancing the inhibitory action of high concen-

trations of ethanol on LH and hCG stimulation of ovarian adenylyl cyclase possibly by a mechanism unrelated to its actions as an inhibitor of proteases. Studies using protease inhibitors included as much as 3% alcohol in the assay (Richert and Ryan, 1977b). Based on our results (Figs. 2, 3), 3% alcohol does inhibit hCG stimulation of ovarian adenylyl cyclase in the presence of protease inhibitors. The results of the present study, however, do not rule out the possibility that at lower concentrations, where ethanol enhances LH and hCG stimulation of adenylyl cyclase activity and where TLCK, in some cases, inhibits LH and hCG stimulation of adenylyl cyclase, TLCK may confer its inhibitory action on cyclase by acting as a protease inhibitor. There is one further aspect of studies with these protease inhibitors that makes published data difficult to interpret. Neither TPCK nor TLCK is soluble in 3% ethanol at the higher concentrations reported to inhibit hCG stimulation of ovarian adenylyl cyclase. Therefore, the actual concentration of the inhibitors in solution in the assay was unknown. Drawing conclusions from such a study on a system as complex as the adenylyl cyclase system would seem tenuous at best.

Based on the findings of the present study, we suggest that the possible role played by proteases in the activation of adenylyl cyclase and the use of synthetic protease inhibitors to study this process requires further study. Although it is possible that proteolytic activation of adenylyl cyclase may be one of the ways by which this enzyme system is activated *in vivo*, we feel it would be premature to suggest a physiologically significant role for proteolytic activation of adenylyl cyclase until all the componenets of the adenylyl cyclase system have been identified and their interactions characterized more extensively.

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